Table S1. Recommended microplate reader parameters

Parameter	Optimized settings	Reason
Plate type	Greiner 96 well, black	Black plates reduce background and fluorescence crosstalk, improves sensitivity. (Z'=0.19 for black U-bottom vs. Z'=0.00 for clear U-bottom plates)
Well shape	V-bottom	Retinal organoids naturally self-center. Simplifies fluorescent plate reads. (Z'=0.33 for black V-bottom vs. Z'=0.19 for black U-bottom plates)
Read mode	Top read	Allows Z-dimensional focus and the use of black v-bottom plates.
Volume	340 µl	Fills well completely, no meniscus.
Flash mode	1 (400 Hz)	A high flash mode increases sensitivity. However if sample viability were a concern (especially if using lower media volumes), a lower flash mode (100 Hz) can be used instead. (Z'=0.33 for 400Hz vs. Z'=0.32 for 100 Hz).
# of flashes	20	Lower flash numbers increase speed (throughput). Higher flash numbers improve accuracy. (See Table S2)

Assay parameters were determined by scanning transgenic GFP-expressing retinal organoids and wild type controls (n=6/condition).

Table S2. Estimated throughput performance

# of flashes	2	5	10	20	50	
Scan time per well	0.1875	0.1979	0.2187	0.2395	0.3229	sec
Scan time per plate	18	19	21	23	31	sec
Estimated daily capacity	2,618	2,541	2,400	2,273	1,878	plates
Estimated daily capacity	251,345	243,952	230,400	218,273	180,313	Individual retinal organoids
SSMD*	>> 2	>> 2	>> 2	>> 2	>> 2	
Z' factor (for n=6)	0.30	0.31	0.32	0.33	0.33	

Estimated daily capacity was calculated adding 15 seconds per 96 well plate to account for handling time between plates. SSMD*: robust SSMD (strictly standardized mean difference).

Table S3. Analytic metrics for evaluating assay quality

Parameter	Formula	Interpretation	HTS compatible values
Signal to background ratio	$S{:}B = \frac{\mu_p}{\mu_n}$	Measures the sensitivity of the assay for signal detection; normally calculated using controls.	>2-fold
Coefficient of variation	$\%CV = \frac{\sigma}{\mu} \times 100$	Represents intra-assay precision or repeatability.	<15%
SSMD*	$\hat{\beta} = \frac{\tilde{X}_p - \tilde{X}_n}{1.4826\sqrt{(\tilde{s}_p)^2 + (\tilde{s}_n)^2}}$	Proposed as a preferred measure of HTS assay quality due to its robustness	$\hat{\beta}$ ≥ 2: excellent quality 2 > $\hat{\beta}$ ≥1: good quality 1 > $\hat{\beta}$ ≥ 0.5: acceptable quality $\hat{\beta}$ < 0.5: poor quality
Z' factor	$Z' = 1 - \frac{(3\,\sigma_p + 3\sigma_n)}{\lvert\mu_p - \mu_n\rvert}$	An accepted, more stringent measure of HTS assay quality. Z' is used in the absence of library compounds to optimize an assay prior to screen.	> 0.5: excellent quality 0 to 0.5: acceptable quality = 0: for "yes/no" type of assay < 0: should be improved

"*p*" and "*n*" correspond to the values of positive and negative controls respectively. μ: mean; σ: standard deviation; SSMD*: robust SSMD (strictly standardized mean difference); $\tilde{X}$: sample median; $\tilde{s}$: sample median absolute deviation. (Described in detail in Inglese at el., 2007; White et al., 2016; and Zhang, 2008, 2011).

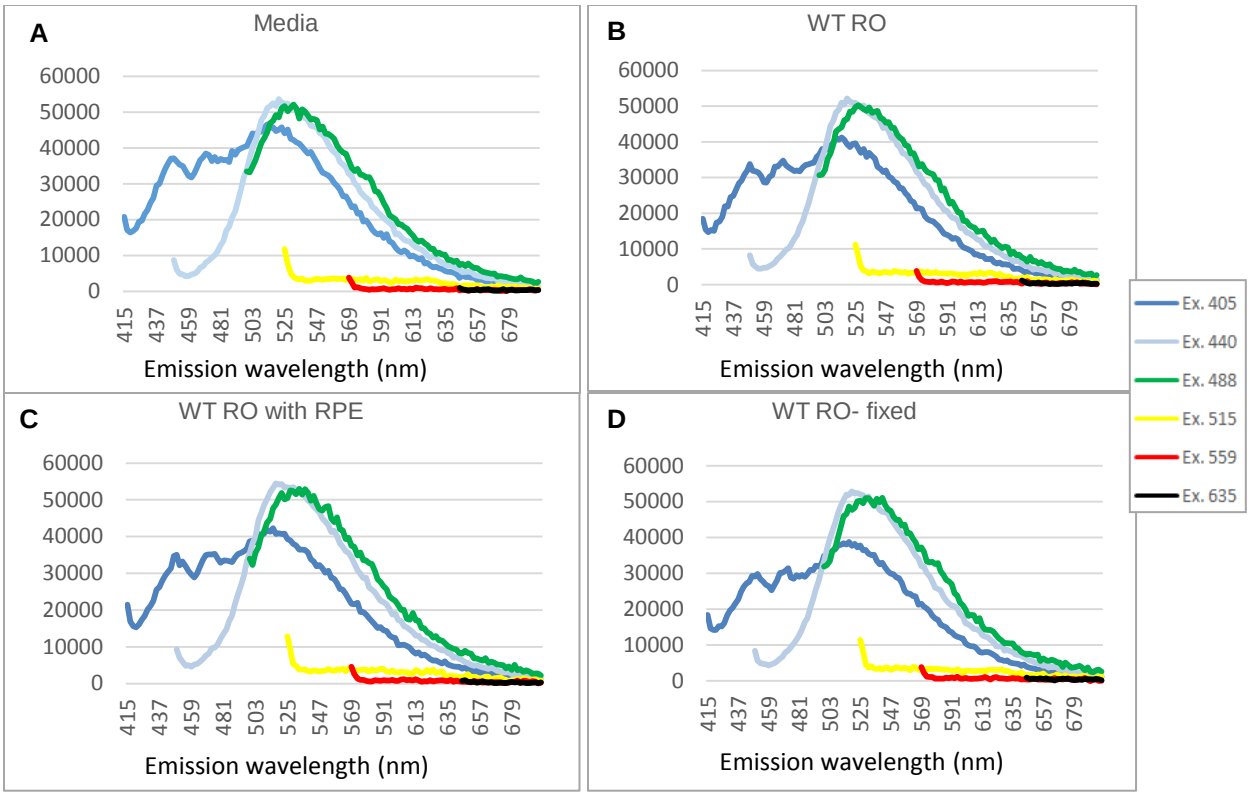

Figure S1. Background fluorescence profiles. (A-D) Graphs show fluorescence emission profiles for common excitation wavelengths (see inset). Fluorescence intensity scans were performed on clear media (A); live, not labeled, wild type retinal organoids (RO, B); retinal organoids with small amounts of RPE attached to the tip (C); and retinal organoids fixed with 4% paraformaldehyde for 10 minutes (D). Curves represent average fluorescence profiles of 5 independent ROs per condition.

A

Fluorophore	Excitation (nm)	Emission (nm)
Hoechst	350/5	450/5
GFP	485/5	510/15
Calcein	492/5	513/5
YFP	514/5	534/10
DiI	551/5	569/5
Bodipy TR	592/5	624/5
Sytox green	504/5	526/5

B

DiI

Ex\Em	560	562	564	566	568	570	572	574	576	578	580
535	670	755	898	782	805	841	931	982	859	702	859
537	777	831	809	974	1016	1028	812	867	716	762	704
539	688	774	685	914	899	858	906	829	823	842	801
541	804	750	972	978	912	1055	924	924	897	837	856
543	1040	895	952	1041	957	1004	971	943	955	896	879
545	851	888	829	1029	941	1078	1061	1082	1033	866	824
547	957	1154	1041	994	1040	1021	1062	1131	1019	919	979
549	1197	1044	1223	1263	1338	1161	1052	1090	1076	978	847
551	1522	1286	1332	1071	1237	1387	1089	1085	1157	807	963
553	3235	1825	1770	1323	1237	1265	1514	1318	1102	1382	1033
555	420898	3905	1999	1582	1512	1513	1412	1426	1141	1085	1060
557	OVER	305033	3443	2269	1726	1412	1373	1142	1152	1046	1049
559	OVER	OVER	61654	3393	1944	1708	1341	1129	1267	1015	1015

Figure S2. Optimized excitation and emission wavelengths. (A) Table shows the optimal excitation and emission wavelengths identified by performing 3D fluorescence scans (i.e. scans spanning a range of excitation and emission wavelengths) on transgenic retinal organoids (GFP and YFP), or retinal organoids stained with different fluorescent dyes (Hoechst, calcein, DiI, Bodipy TR, Sytox green). Live organoids were used for this experiment, with the exception of Sytox green, which was used on fixed organoids. Results are expressed as "wavelength/bandwidth". (B) Example of a 3D scan performed for one of the conditions (DiI staining). Heat map is a visual representation used to aid in the determination of optimal parameters. Lowest wavelength values (blue) provide decreased sensitivity, whereas highest values (red) constitute an artifact from the overlap of the excitation an emission spectral curves. Box illustrates the wavelengths that were chosen for their sensitivity performance among the mid ranges of the matrix (green color set at the 50th percentile).

SUPPLEMENTARY METHODS

Generation of transgenic hiPSC lines

The human episomal CB-iPSC6.2 line (A18945, ThermoFisher Scientific; Burridge et al., 2011) was maintained on Matrigel (growth-factor-reduced; BD Biosciences)-coated plates with mTeSR1 medium (Stemcell Technologies). Transgenic cell lines were generated by electroporation with the Neon Transfection System (Invitrogen) according to manufacturer instructions as described in Ranganathan et al., 2014. The following plasmid DNAs were used: i) CRISPR/Cas9-mediated constitutively expressed GFP hiPSC line: 1µg of AAV-CAGGS-EGFP donor vector (Addgene # 22212, gift from Rudolf Jaenisch; Hockemeyer et al., 2009); 0.6 µg of pCas9_GFP (Addgene # 44719, gift from Kiran Musunuru); and 0.3 µg of gRNA_AAVS1-T2 (Addgene # 41818, gift from George Church; Mali et al., 2013); ii) PiggyBac transposon-mediated constitutively expressed membrane YFP hiPSC line: 1 µg of PB-myr-tagged YFP donor vector and 0.3 µg of PB-HA transposase expression vector (Wellcome Trust Sanger Institute; Cadiñanos et al., 2007).

Briefly, cells were pre-treated with 5 µM blebbistatin for 24h to increase cell viability, followed by treatment with Accutase (Stemcell Technologies) for 5min, dissociation into single cells, centrifugation at 80 x g for 5 min. for pellet formation and incubation on ice for 15 min. The corresponding plasmids for each transgenic cell line were combined in R buffer, resuspended in the plasmid cocktail and electroporated with a 10 µl tip-type and the following parameters: 1,300 V; 20 ms pulse length; 1 pulse. Cells were then gently resuspended into 1ml of mTeSR1 plus 5µM blebbistatin, incubated at room temperature for 20 min and plated onto Matrigel-coated 35mm TC treated dishes containing mTeSR1 and 5 µM blebbistatin. Finally, cells were incubated at room temperature for 20 min and cultured thereafter in 37 C and 5% CO2. After 5 days, stable clonal sublines were manually selected with a Leica MZ-16F fluorescence stereomicroscope.

Media and dye solutions for staining of retinal organoids

Fluorescent staining and scanning of retinal organoids was performed in clear retinal differentiation medium (cRDM) containing 60% DMEM/F12 no phenol red (cat# 21041-025), and 40% FluoroBrite DMEM (cat# A18967-01), supplemented with 1x B27 (cat# 12587-010), 0.4x Glutamax (cat# 35050-079), 1x MEM-Non essential amino acids (cat# 11140-050), and 1x antibiotic-antimycotic (cat# 15240-062), all from Thermo Fisher Scientific. Solutions of the corresponding dyes were prepared in cRDM as follows: Hoechst 33342 (cat# H3570), 2.5 µl/ml; Calcein AM 4mM stock solution in DMSO (cat# C34852), 2.75 µl/ml ; Vybrant CM-DiI (cat# V-22888), 5 µl/ml; and Bodipy TR (cat# C34556), 20 µl/ml; Sytox green 5 mM (cat# S7020), 2 µl/ml (all from Thermo Fisher Scientific). Staining was performed as described in Methods. Long-term suspension culture media for aged retinal organoids consists of DMEM/F12 -Glutamax (cat#10565018, ThermoFisher Scientific) supplemented with 1% N2 (cat# 17502048, ThermoFisher Scientific), 1x MEM-Non essential amino acids, 1% antibiotic–antimycotic, 100 µM Taurine (cat# T0625, Sigma-Aldrich), and 10% fetal bovine serum (cat# S11150, Atlanta Biologicals), as described in Zhong et al., 2014.

Whole-mount immunofluorescence

Retinal organoids were fixed in 4% paraformaldehyde for 10 min and rinsed 3X in PBS, followed by 3X 20 min washes in PBST (0.25% Triton X-100 in PBS) with rocking.

For Otx2 immunofluorescence organoids were blocked in 10% NGS (normal goat serum), 0.25% PBST, overnight at room temperature. They were then washed 3X 30 min in 0.25% PBST, and 3X 30 min in PBS, followed by incubation in rabbit-anti-Otx2 antibody (cat# AB9566; ThermoFisher Scientific) diluted 1/500 in 2% NGS, 0.25% PBST for 2 days at room temp with rocking. Subsequently, organoids were rinsed 3X 30 min in 0.25% PBST, and 3X 30 min in PBS, followed by incubation with secondary antibody: goat-anti-mouse Alexafluor 514 (cat# A-31558; ThermoFisher Scientific) , diluted 1/500, in 2%NGS, 0.25% PBST, for 2 days at room temp with rocking. Finally, they were washed 3X 30 min in

0.25% PBST, and 3X 30 min in PBS. For size normalization organoids were counterstained by incubation with Bodipy TR, 20 µl/ml at 37°C for 90 min, followed by 3 washes in cRDM, and plated in black v-bottom 96-well plates for scanning.

For Pou4f2 immunofluorescence organoids were blocked in 10% NDS (normal donkey serum), 0.25% PBST, overnight at 4°C. They were then washed 3X 30 min in 0.25% PBST, and 3X 30 min in PBS, followed by incubation in goat-anti-Brn3b(Pou4f2) antibody (Santa Cruz Biotechnologies, cat# SC-6026x) diluted 1/1000 in 2% NDS, 0.25% PBST for 3 days at 4°C with rocking. Subsequently, organoids were rinsed 3X 30 min in 0.25% PBST, and 3X 30 min in PBS, followed by incubation with secondary antibody: donkey-anti-goat-Alexafluor 546 (ThermoFisher Scientific, cat# A-11056), diluted 1/500, in 2%NDS, 0.25% PBST, for 3 days at 4°C with rocking. Finally, they were washed 3X 30 min in 0.25% PBST, and 3X 30 min in PBS. For size normalization organoids were counterstained by incubation with Sytox green, 10 µM at 37°C for 90 min, followed by 3 washes in cRDM, and plated in black v-bottom 96-well plates for scanning.

Immunofluorescence on cryosections

Retinal organoids were fixed in 4% paraformaldehyde for 10 min and rinsed 3X in PBS, followed by sucrose gradient and embedding in a 1:1 mixture of 25% sucrose:OCT. 12 µm thick sections were obtained using a cryostat, and slides were incubated at room temperature for 60 minutes, followed by PBS wash and blocking in 10% NGS, 0.25% PBST for 1 hour. Slides were then incubated overnight at 4°C in anti-recoverin antibody (cat# AB5585, Millipore- ThermoFisher Scientific) diluted 1/500 in 2% NGS, 0.25% PBST. After 3X 10 min. washes in PBS, slides were incubated in secondary antibody: goat-anti-rabbit Alexafluor 546 (cat# A-11035; ThermoFisher Scientific), diluted 1/500, in 2%NGS, 0.25% PBST, for 2 hrs. at room temp. Finally, they were washed 3X 10 min in PBS and incubated for 5 min. in DAPI (cat# D1306; ThermoFisher Scientific) for nuclear counterstaining. Imaging was performed using a Zeiss LSM 510 confocal microscope.

Eqn. S1. Size normalization

$$Norm.X_a = \frac{X_a * \overline{Y}}{Y_a}$$

$Norm.X_a$: Normalized fluorescence intensity value of fluorophore X for organoid a.
X_a: Fluorescence intensity value of fluorophore X for organoid a.
Y_a: Fluorescence intensity value of global fluorophore Y (normalizer) for organoid a.
$\overline{Y}$: Mean of fluorescence intensity values for fluorophore Y.

SUPPLEMENTARY REFERENCES

Hockemeyer, D., Soldner, F., Beard, C., Gao, Q., Mitalipova, M., Dekelver, R.C., Katibah, G.E., Amora, R., Boydston, E.A., Zeitler, B., Meng, X., Miller, J.C., Zhang, L., Rebar, E.J., Gregory, P.D., Urnov, F.D., Jaenisch, R. (2009). Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat. Biotechnol.* **27**, 851-7.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., Dicarlo, J.E., Norville, J.E., Church, G.M. (2013). RNA-Guided Human Genome Engineering via Cas9. *Science* **339**, 823-6.

Ranganathan, V., Wahlin, K., Maruotti, J., Zack, D.J. (2014). Expansion of the CRISPR–Cas9 genome targeting space through the use of H1 promoter-expressed guide RNAs. *Nat. Commun*. **5**, 4516.

Cadiñanos, J. and Bradley, A. (2007). Generation of an inducible and optimized *piggyBac* transposon system. *Nucleic Acids Res.* **35,** e87.